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TITLE: Priming the Tumor Immune Microenvironment Improves Immune Surveillance of Cancer Stem Cells and Prevents Cancer Recurrence

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13. SUPPLEMENTARY NOTES					
14. ABSTRACT We were able to complete Specific Aim #1 of our two-aim application by extending our preliminary data. Thus, we demonstrated that our targeted elimination of cancer associated fibroblasts (CAFs) with a pFap vaccine in combination with doxorubicin (Dox) chemotherapy can prevent tumor-induced immune suppression. This, in turn, enhanced endogenous anti-tumor immunity, resulting in suppression of spontaneous metastasis and increased life span. Our findings re-emphasize the important contribution of CAFs during tumor progression, specifically during the development of tumor-mediated immune suppression. Collectively, these data add to the growing evidence supporting an integrate role for the tumor microenvironment (TME) during this process which ultimately leads to tumor cell escape from clearance by the host's immune system. Importantly, our data obtained thus far re-enforce the validity and importance of CAFs as a therapeutic target for metastatic breast cancer.					
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INTRODUCTION:

The major hypothesis driving this proposal is that modulating the immune tumor micro-environment (TME) can improve immune surveillance of breast cancer stem cells (CSCs) and prevent cancer recurrence. This will be achieved by priming the TME by our pFap/DOX combination therapy, followed by boosting of anti-CSC immune responses by treatment with immuno/chemotherapy targeted to CSCs.

BODY:

Aim #1 of this 2-Aim 2-year application was completed as it successfully tested the hypothesis that priming of the tumor immune microenvironment (TME) by our combination therapy of a DNA vaccine against fibroblast activation protein (FAP), overexpressed on cancer-associated fibroblasts (CAFs) plus DOX chemotherapy enhanced breast tumor-immune responses in a mouse model system. Specifically, we achieved the following:

- 1) Elimination of CAFs improved the anti-tumor effects of DOX in a syngeneic mouse breast cancer model. Briefly, Balb/c mice were vaccinated orally in a prophylactic setting with either empty vector as a control or pFap DNA vaccine carried by attenuated *Salmonella typhimurium*, followed by orthotopic challenge with 5×10^3 4T1 breast tumor cells and treatment with DOX (Fig. 1A),. Control animals exhibited rapid tumor growth and palpable tumors within 11d post injection. In contrast, our combination therapy retarded primary tumor growth (Fig. 1B) and significantly reduced tumor weight (Fig. 1C), and also was more effective than either treatment alone.
- 2) Our combination therapy also effectively altered the TME as it reduced tumor expression of growth factors (Vegf, Pdgf) and cytokine (GM-CSF). These factors are implicated in tumor-mediated immune suppression. Thus, qRTPRC analysis of mRNA of primary tumors of mice treated in a prophylactic setting revealed high expression in the tumor stroma (Figs. 2A, B, C) which was, however, significantly reduced after combination therapy.
- 3) Our combination therapy increased Th1 cytokines but reduced Th2 cytokines resulting in enhanced CTL activation in primary tumors. This shift in polarization of the TME from a pro-tumor Th2 response to an anti-tumor Th1 response was significant and it resulted in increased activation of CTLs. This was documented by immunoblotting of tumor extracts indicating reduced protein levels of IL-6 and IL-4 (Fig. 3A) and increase in levels of IL-2 and IL-7 (Fig. 3B) culminating in the increased relative percentage of activated CTLs in primary tumors (Fig. 3C).
- 4) We demonstrated that the combination therapy enhances endogenous anti-tumor specific CTL responses. The combination therapy decreased immune suppressor cells and Th2 cytokines and increased immune effector cells as well as Th1 cytokines in the TME. Consequently, we determined whether these changes translated into enhanced endogenous anti-tumor specific CTL responses. Initially, we isolated splenocytes from tumor bearing mice treated with the combination therapy and measured proliferation and activation of CTLs after exposure to tumor cells ex vivo

- (Figs. 4A, B). Additionally, we performed flow cytometry analysis showing that our combination therapy could significantly increase the percentage of tumor cells in the early apoptotic state compared to tumors from untreated mice or mice treated with only a single therapy (Fig. 4C).
- 5) We demonstrated in a therapeutic setting that our combination therapy could suppress spontaneous breast cancer metastasis resulting in increased life span of experimental animals. In fact, our quite challenging therapeutic setting, where established tumors and their spontaneous lung metastases are treated by our combination therapy, actually more closely recapitulates the actual clinical treatment of breast cancer than a prophylactic setting. Thus, mice were first challenged orthotopically with 4T1 tumor cells and primary tumors allowed to grow to a size of 500mm³, prior to vaccination with pFap, resection of the primary tumor, and treatment with DOX chemotherapy (Fig. 5A). Two booster vaccinations of pFap, prior to tumor resection, did not significantly alter primary tumor weight or size (Fig. 5B). Importantly, however, the combination therapy did achieve a significant increase in life span, compared to control animals (Fig. 5C; $p < 0.05$). Furthermore, lungs from mice treated with our combination therapy, show a significant reduction in metastatic foci in contrast to untreated mice, revealing extensive growth of large metastatic foci (Fig. 5D). In fact, estimations of metastatic surface area indicated that the combination therapy significantly reduced the metastatic load compared to all control groups of animals (Fig. 5E; $p < 0.0005$).

KEY RESEARCH ACCOMPLISHMENTS:

- Combined therapy of DNA vaccine against FAP plus DOX chemotherapy suppresses breast tumor growth.
- Combination therapy increased Th1 cytokines but reduced Th2 cytokines resulting in enhanced CTL activation.
- Anti-tumor specific responses altered the TME as it reduced tumor expression of growth factors Vegf and Pdgf as well as cytokine GM-CSF.
- In a therapeutic setting, combination therapy suppressed spontaneous breast cancer metastasis and increased life span of mice.

To our knowledge, we are first to demonstrate that a DNA vaccine designed from targeted *in vivo* elimination of CAFs, when combined with DOX chemotherapy, can restore anti-tumor immunity leading to suppression of breast tumor growth and spontaneous metastasis.

REPORTABLE OUTCOMES:

Poster presentation (p4-20) at Era of Hope meeting at Orlando, FL in Tumor Immunology session on 8/3/11. The presentation was entitled “Cancer associated fibroblasts modulate the tumor immune microenvironment to promote tumor growth and metastasis in a mouse model of breast cancer”.

Solid tumors are multi-cellular tissues comprised of tumor cells and stromal cells, including fibroblasts, endothelial and inflammatory cells residing within the tumor microenvironment (TME). Recent studies focusing on cancer-associated fibroblasts (CAFs) have uncovered a prominent role for these cells in promoting tumor growth and progression. CAFs constitute the major cell type in the tumor stroma and are characterized by expression of the transmembrane serine protease Fibroblast-activation Protein (FAP). Tumor associated stroma differs from stroma in normal tissue in that it has an activated phenotype characterized by production of certain extracellular matrix components, growth factors and cytokines. Additionally, local inflammation associated with solid tumors commonly results from factors released by the tumor stroma and promotes tumor progression. CAFs are thought to play a prominent role in these tumor-promoting processes.

We utilized the FAP antigen as a novel target for a DNA vaccine against CAFs and tested its efficacy in combination with doxorubicin in a mouse model of breast cancer. Our findings showed that: 1) a DNA vaccine targeted to FAP can specifically and effectively eliminate CAFs *in vivo*, 2) targeting CAFs improves cancer chemotherapy by increasing drug uptake, and 3) targeting CAFs, in combination with doxorubicin chemotherapy, modulates the tumor immune microenvironment from a pro-tumor Th2 to anti-tumor Th1 polarization. Oral vaccination with pFAP generated a CD8⁺ T cell dependent anti-tumor response against CAFs, which resulted in their elimination *in vivo* and lead to a reduction of collagen type I in the stroma of primary tumors. When combined with chemotherapy, this decrease in collagen expression resulted in improved uptake of doxorubicin by primary tumors, which improved its anti-tumor effects. Additionally, we found that our combination therapy also resulted in modulation of the immune TME from a pro-tumor Th2 to an anti-tumor Th1 polarization. This shift in immune polarization was characterized by increased protein expression of IL-2 and IL-7, suppressed recruitment of tumor-associated macrophages, myeloid derived suppressor cells, T regulatory cells, and decreased tumor angiogenesis and lymphangiogenesis. Additionally, the vaccine improved the anti-metastatic effects of doxorubicin chemotherapy and enhanced suppression of IL-6 and IL-4 protein expression while increasing recruitment of dendritic cells and CD8⁺ T cells. We also found that CD8⁺ splenocytes isolated from mice treated with both pFAP and doxorubicin exhibited enhanced proliferation and activation in response to stimulation with tumor cells *in vitro*. Our findings demonstrate that CAFs promote tumor growth and metastasis through their role as key modulators of immune polarization in the TME and are valid targets for improved immune therapy of metastatic breast cancer.

CONCLUSION:

We completed Aim #1 of this 2-Aim, 2-year application. To our knowledge, we are first to conclusively demonstrate that a DNA vaccine against fibroblast activation protein (FAP), overexpressed on cancer-associated fibroblasts (CAFs), combined with DOX chemotherapy, eliminated CAFs and restored anti-tumor immunity in the tumor microenvironment (TME). This novel strategy lead to statistically significant suppression of breast tumor growth and spontaneous metastasis in a mouse breast tumor model.

APPENDICES:

Figure Legends:

Fig. 1 Combination therapy effectively suppresses primary tumor growth.

(A) Mice were challenged in a prophylactic setting. (B) Tumor dimensions were measured twice per week and used to calculate the tumor volume. *, $p < 0.05$, compared with empty vector. (C) 25 d after tumor cell challenge, primary tumors were resected and their weight (TW) compared to body weight (BW) to calculate tumor burden.

Fig. 2 Combination therapy reduces tumor associated Vegfa, GM-CSF, and Pdgfc mRNA and protein expression.

Mice were challenged in a prophylactic setting and primary tumors were isolated 25 d later. Total RNA was isolated from the stroma (S) and tumor cells (TC) by laser capture dissection microscopy and used to generate cDNA for qRT-PCR analysis. Gene expression is normalized to actin and shown relative to empty vector stroma for Vegfa, GM-CSF and Pdgfc (A-C, left panel, respectively). *, $p < 0.05$ **, $p < 0.005$. Whole cell extracts were derived from primary tumors and subjected to immunoblotting to detect VEGF, GM-CSF and Pdgfc protein (A-C, right panel, respectively).

Fig. 3 Combination therapy reduces Th2 and increases Th1 cytokine protein expression resulting in increased CTL activation in primary tumors.

Mice were challenged in a prophylactic setting and primary tumors isolated 25 d later. (A-B) Tumor homogenates were analyzed by Western blotting to determine Th2 cytokine (A) and Th1 cytokine (B) protein levels. (C) Live primary tumor cell suspensions were analyzed by flow cytometry using anti-CD8 and anti-CD25 antibodies to detect activated T-cells. Results are shown as percent of $CD8^+/CD25^+$ T cells relative to mice treated with the combination therapy.

Fig. 4 Combination therapy results in enhanced endogenous anti-tumor CTL responses.

Splenocytes isolated from tumor bearing mice treated with the combination therapy were stimulated with irradiated 4T1 cells *ex vivo*. Cultures were then analyzed by flow cytometry using the following antibody combinations: (A) CD8 to detect CTLs, (B) CD8/CD25 to detect activated CTLs. Primary tumors were isolated from mice 10 d after the final doxorubicin treatment and the percentage of early apoptotic cells in live primary tumor cell suspensions was quantified by flow cytometry using Annexin V and propidium iodide staining. (C)

Fig. 5 Combination therapy suppresses spontaneous metastasis and increases lifespan of mice treated in a therapeutic setting

(A) Primary tumors were allowed to establish prior to resection and treatment with the combination therapy. (B) Booster vaccination with pFap 4 d prior to tumor resection did

not significantly affect primary tumor size. (C) Kaplan-Meier survival curves of mice after resection of their primary tumor. *, $p < 0.05$. (D) Lungs were isolated from moribund mice, sectioned, and stained with hematoxylin and eosin to visualize metastatic foci. Scale bar=1mm. (E) Surface areas (SA) of metastatic foci and lung were measured using ImageJ software (n-5 mice/group). Results are depicted as percent $SA^{\text{metastasis}}/SA^{\text{lung}}$. ***, $p < 0.0005$.

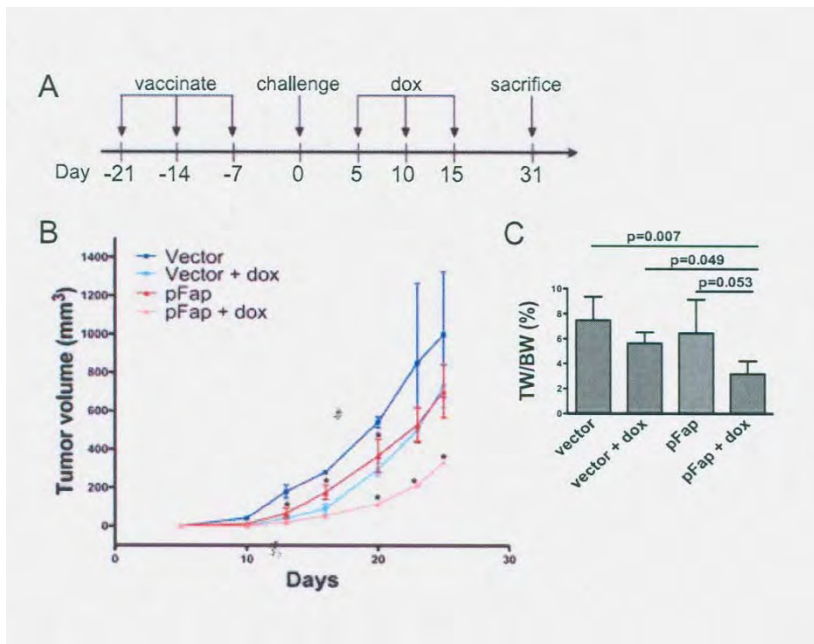


Fig 1. Combination therapy effectively suppresses primary tumor growth.

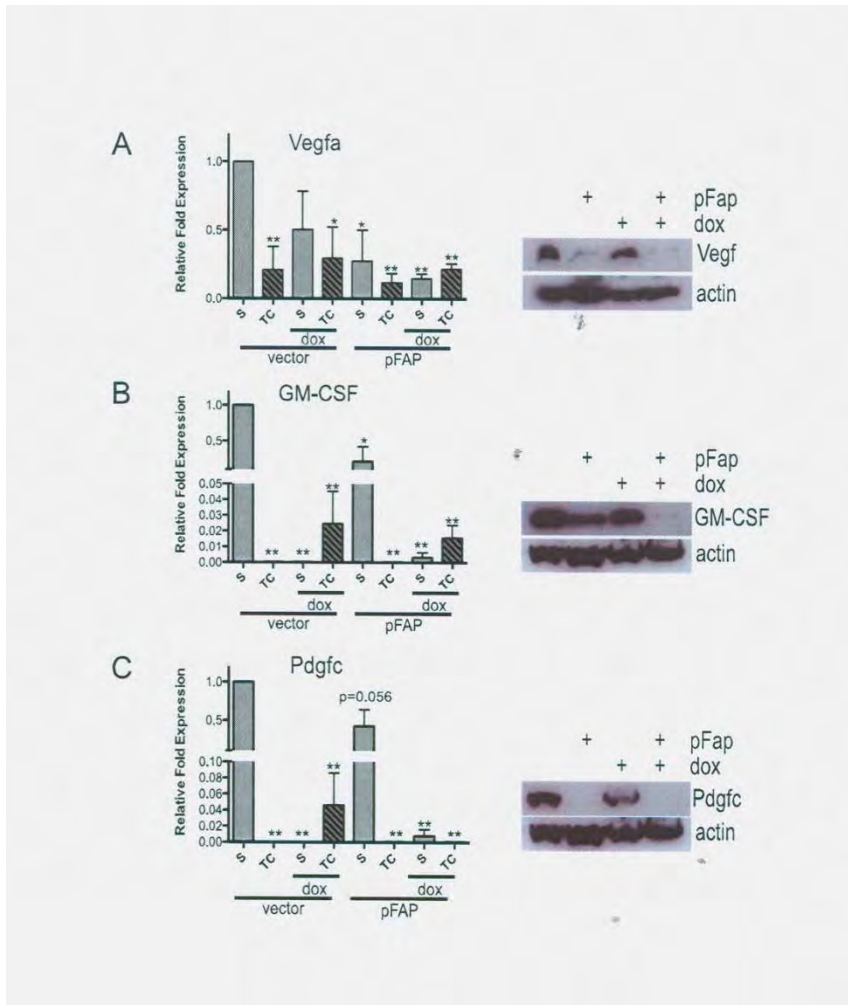


Fig 2. Combination therapy reduces tumor associated Vegfa, GM-CSF, and Pdgfc mRNA and protein expression.

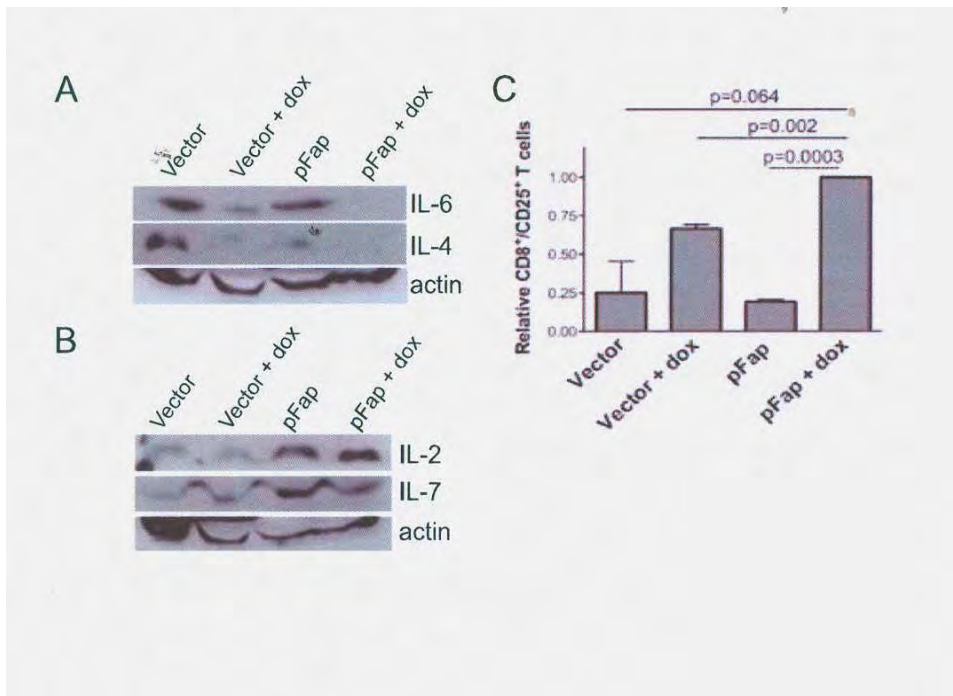


Fig 3. Combination therapy reduces Th2 and increases Th1 cytokine protein expression resulting in increased CTL activation in primary tumors.

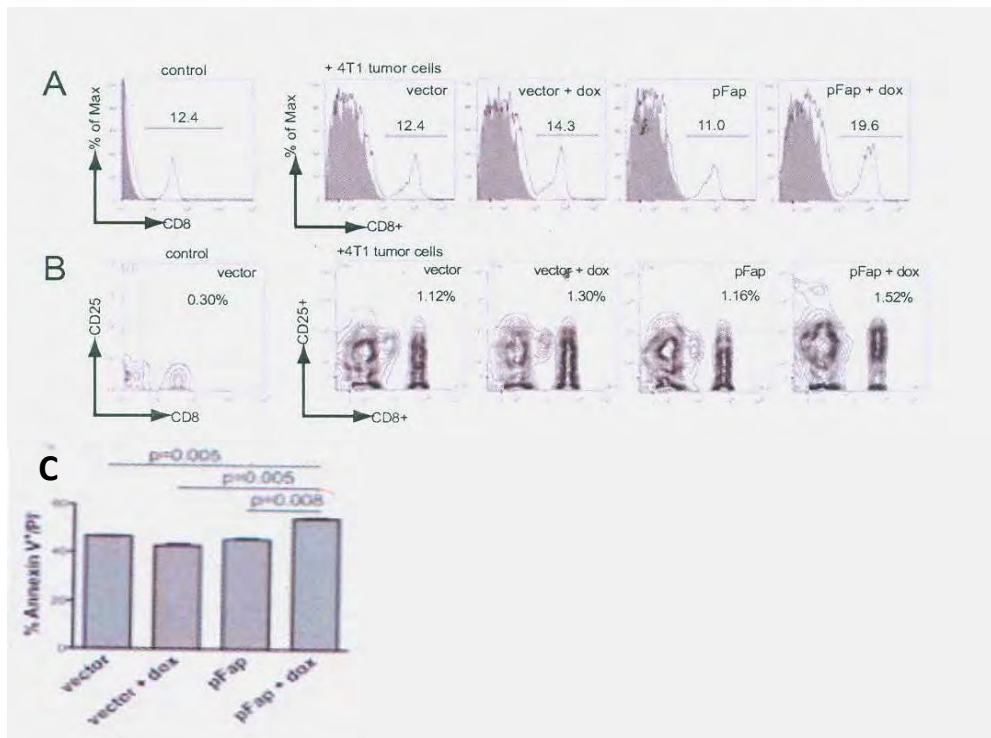


Fig 4. Combination therapy results in enhanced endogenous anti-tumor CTL responses.

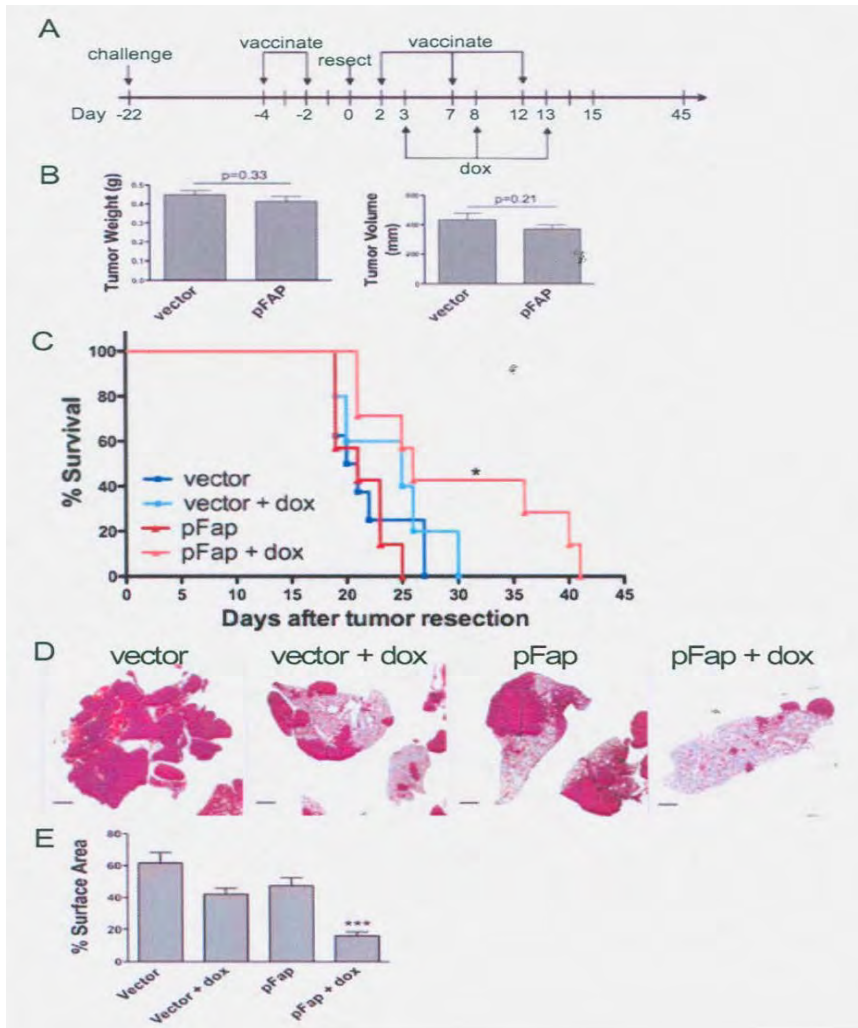


Fig 5. Combination therapy suppresses spontaneous metastasis and increases lifespan of mice treated in a therapeutic setting